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Original Article

Regional Differences in Prevalence of Macrolide Resistance among Pediatric *Mycoplasma pneumoniae* Infections in Hokkaido, Japan

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SUMMARY: Recently, macrolide-resistant (MR) *Mycoplasma pneumonia* appeared, and prevalence of macrolide resistance among *M. pneumoniae* infections varies by country. However, reports on regional differences in the prevalence of MR *M. pneumonia* within a country are scarce. In this study, 617 nasopharyngeal swab samples were collected from 617 pediatric patients, and DNA of *M. pneumoniae* was identified in 95 samples. In 51 of the 95 *M. pneumonia* positive samples, we detected the presence of mutation A2063G mutation (conferring macrolide resistance) in the 23S rRNA gene. The overall macrolide resistance rate was 53.7%, but there were regional differences: 0.0% in Muroran, 5.3% in Asahikawa, 55.3% in Sapporo, and 100.0% in Kushiro. Statistically significant pairwise differences in the prevalence of MR *M. pneumoniae* were observed among these cities except for the pair of Muroran and Asahikawa. After exclusion (in order to avoid the influence of macrolides) of patients who were prescribed macrolides before collection of nasopharyngeal swab samples, statistically significant differences persisted: 0.0% in Muroran, 5.6% in Asahikawa, 38.5% in Sapporo, and 100.0% in Kushiro.

INTRODUCTION

Mycoplasma pneumoniae is a common causative pathogen of community-acquired respiratory tract infections mainly in children and young adults (1). Macrolides are generally considered the drugs of choice for treatment of children with *M. pneumoniae* infection (2). Since approximately the year 2000, macrolide-resistant (MR) *M. pneumoniae* has been appearing in Asia, Europe, Canada, and the USA (3–14). The rate of macrolide resistance among *M. pneumoniae* infections ranges from 3 to 26% in Europe (9,15), from 63 to 97% in China (16–19), and from 25 to 93% in Japan (20–22). The total number of febrile days and the number of febrile days during macrolide administration are greater in patients infected with MR *M. pneumoniae* than in patients infected with macrolide-sensitive (MS) *M. pneumoniae* (23). Therefore, it is important to understand the regional prevalence of MR *M. pneumoniae* infections to predict the duration of fever caused by *M. pneumoniae*. Differences in the prevalence of MR *M. pneumoniae* among 7 surveillance areas throughout Japan were recently reported (21), but such differences among cities are not known. The purpose of this study was to determine the differences in prevalence of MR *M. pneumonia* among 4 cities in Hokkaido (83,457 km^2), the northernmost island of Japan.

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MATERIALS AND METHODS

Clinical samples: Nasopharyngeal swab samples were collected from pediatric patients who were suspected of having a respiratory tract infection associated with *M. pneumoniae* from December 1, 2012, to July 31, 2014, at 8 pediatric clinics and in the department of pediatrics of 6 hospitals in the cities of Sapporo, Asahikawa, Kushiro, and Muroran, in Hokkaido, Japan (Fig. 1). The nasopharyngeal swab samples were suspended in 3 ml of BD universal viral transport medium (Becton Dickinson, Sparks, MD, USA) before extraction of DNA.

Real-time PCR assay: DNA was extracted using the QIAamp DNA mini kit (Qiagen, Venlo, The Netherlands) from 1 ml of BD universal viral transport medium and was finally resuspended in 50 μl of buffer. DNA of *M. pneumoniae* was identified by real-time PCR using Mp181-F and Mp181-R primers and the Mp181-P probe with 1 μl of DNA as described elsewhere (24).

Fig. 1. The locations of 4 cities (Sapporo, Asahikawa, Kushiro, and Muroran) in Hokkaido are shown.

Detection of a resistance-associated point mutation in domain V of 23S rRNA: Mutations associated with resistance to macrolides at sites 2063, 2064, and 2617 in the *M. pneumoniae* 23S rRNA gene (domain V region) were detected by a sequencing method described elsewhere (25). *M. pneumonia* with a point mutation in domain V of the 23S rRNA gene was defined as MR *M. pneumoniae*.

Isolation and molecular typing of *M. pneumoniae* strains: The modified Hayflick medium was used for the isolation of *M. pneumoniae* from patients (26). The *p*1 gene, encoding P1 cytadhesin, an essential pathogenic factor of *M. pneumoniae*, was subtyped by a PCR-based method (27).

Statistical analysis and ethics: All statistical analyses were performed in the JMP software, version 11.0.0 (SAS Institute, Cary, NC, USA). The prevalence rates of MR *M. pneumoniae* were compared by Fisher's exact test. The multiplicity was adjusted by Bonferroni's correction method (adjusted significance level was 0.008 if all combinations of 4 cities were compared). All of the necessary ethics approvals for this study were obtained from the Institutional Review Board of Hokkaido University Hospital for Clinical Research.

RESULTS

A total of 617 nasopharyngeal swab samples were collected from 617 patients, and DNA of *M. pneumoniae* was identified in 95 samples. The average age of the patients was 8.4 years, and the ratio of men to women was 50:45. In 51 of the 95 *M. pneumoniae*-positive samples, the presence of the A2063G mutation in the 23S rRNA gene, a point mutation known to confer macrolide resistance to *M. pneumoniae*, was detected, but other mutations (A2063C, A2063T, A2064G, or C2617G) were not detected. In the remaining 44 *M. pneumoniae*-positive samples, these mutations were not detected. The overall macrolide resistance rate was 53.7% (51 of 95), whereas the municipal resistance rate varied: 0.0% (0 of 9) in Muroran, 5.3% (1 of 19) in Asahikawa, 55.3% (21 of 38) in Sapporo, and 100.0% (29 of 29) in Kushiro (Table 1). Table 1 shows the *P* values of Fisher's exact test for pairwise comparisons of the prevalence rates of macrolide resistance among *M. pneumonia* infections between two cities. Statistical significance was observed for pairwise differences in the prevalence of MR *M. pneumoniae* except for the pair Asahikawa and Muroran. Differences in prevalence of MR *M. pneumoniae* were also observed between patients visiting hospitals and those visiting clinics and between outpatients and inpatients (Table 2). Statistical significance was observed for the differences in the prevalence of MR *M. pneumoniae* between patients with and without macrolide pre-administration: 92.0% (23 of 25) and 40.0% (28 of 70), respectively (Table 3). Macrolides had been administered to 25 (26.3%) of the patients before collection of nasopharyngeal swab samples. After exclusion of the nasopharyngeal swab samples from 25 patients who had received macrolides, statistical significance of the differences was still observed between regions (Table 1) and between patients visiting hospitals and those visiting clinics, whereas the statistical significance of the difference between outpatients and inpatients disappeared (Table 4).

Twenty-three strains of *M. pneumoniae* were found in 23 randomly selected samples, and these were genotyped. Four of 6 strains of MR *M. pneumoniae* from the Kushiro samples were found to be subtype 1, and 2 were variant 2c (Table 5).

DISCUSSION

In the present study, DNA of *M. pneumoniae* was identified in 95 of 617 nasopharyngeal swab samples from patients who were suspected of having respiratory

Table 1. Prevalence of macrolide-resistant *M. pneumoniae* in 4 cities

City	Medical institution	Hospital /Clinic	Total case		Not pre-administrated[1)]		
			No. of MR /total isolates (%)	No. of MR/total in the city (%)	No. of MR /total isolates (%)	No. of MR/total in the city (%)	
Sapporo	A	Hospital	3/5 (60.0)	21/38 (55.3)	2/4 (50.0)	10/26 (38.5)	2) **
	B	Hospital	1/6 (16.7)		1/5 (20.0)		
	C	Hospital	9/11 (81.8)		4/6 (66.6)		
	D	Hospital	5/6 (83.3)		1/2 (83.3)		
	E	Clinic	2/3 (66.7)		2/3 (66.7)		
	F	Clinic	0/1 (0.0)		0/1 (0.0)		
	G	Clinic	0/1 (0.0)		0/1 (0.0)		
	H	Clinic	0/1 (0.0)		0/1 (0.0)		
	I	Clinic	1/2 (50.0)		0/1 (0.0)		
	J	Clinic	0/2 (0.0)		0/2 (0.0)		
Asahikawa	K	Hospital	1/5 (20.0)	1/19 (5.3)	1/5 (20.0)	1/18 (5.6)	*
	L	Clinic	0/14 (0.0)		0/13 (0.0)		**
Kushiro	M	Hospital	29/29 (100.0)	29/29 (100.0)	17/17 (100.0)	17/17 (100.0)	**
Muroran	N	Clinic	0/9 (0.0)	0/9 (0.0)	0/9 (0.0)	0/9 (0.0)	
TOTAL			51/95 (53.7)		28/70 (40.0)		

MR, macrolide resistant.
1): Samples from patients in whom macrolides were not pre-administrated.
2): Similar statistically significances were observed in total cases and cases not pre-administrated.
*, *P* value by Fisher's exact test, <0.05. **, *P* value by Fisher's exact test, <0.008.

Table 2. Prevalence of macrolide-resistant *M. pneumoniae*

Case	Total No.	Macrolide		*P* value[1)]
		Sensitive (%)	Resistant (%)	
Hospitals	62	14 (22.6)	48 (77.4)	<0.0001*
Clinics	33	30 (90.9)	3 (9.1)	
Outpatients	70	39 (55.7)	31 (44.3)	0.0024*
Inpatients	25	5 (20.0)	20 (80.0)	

1): *P* value by Fisher's exact test. *, statistically significant.

Table 3. Prevalence of macrolide-resistant *M. pneumoniae* in patients pre-administered macrolide

Pre-administration	Total No.	Macrolide		*P* value[1)]
		Sensitive (%)	Resistant (%)	
Yes	25	2 (8.0)	23 (92.0)	<0.0001*
No	70	42 (60.0)	28 (40.0)	

1): *P* value by Fisher's exact test. *, statistically significant.

tract infections associated with *M. pneumoniae*. Although the overall prevalence of macrolide resistance among *M. pneumoniae* infections was 53.7%, substantial regional differences were observed: high MR rate in Kushiro (100.0%) and low MR rates in Asahikawa (5.3%) and Muroran (0.0%). Differences in the prevalence of MR *M. pneumonia* among prefectures in Japan have been reported previously (21). In the present report, we showed for the first time the differences in the prevalence of macrolide resistance among *M. pneumoniae* infections across cities in one prefecture of Japan. Information on the prevalence of MR *M. pneumoniae* in each city is important for clinicians because the mean prevalence of 53.7% is far from local prevalence rates in Kushiro, Asahikawa, and Muroran.

Table 4. Prevalence of macrolide-resistant *M. pneumoniae* in whom macrolides were not pre-administered

Case	Total No.	Macrolide		*P* value[1)]
		Sensitive (%)	Resistant (%)	
Hospitals	39	13 (33.3)	26 (66.7)	<0.0001*
Clinics	31	29 (93.6)	2 (6.4)	
Outpatients	60	38 (63.3)	22 (36.7)	0.1833
Inpatients	10	4 (40.0)	6 (60.0)	

1): *P* value by Fisher's exact test. *, statistically significant.

Table 5. *p*1 gene typing of *M. pneumoniae* isolates

City	Macrolide -sensitive (S)/ -resistant (R)	No. tested	*p*1 gene typing			
			subtype 1	subtype 2	variant 2a	variant 2c
Sapporo	S	3	1	0	0	2
	R	5	5	0	0	0
Asahikawa	S	3	3	0	0	0
	R	0	0	0	0	0
Kushiro	S	0	0	0	0	0
	R	6	4	0	0	2
Muroran	S	6	0	1	3	2
	R	0	0	0	0	0
TOTAL		23	13	1	3	6

The fact that the number of clinics and hospitals that participated in this study was limited raises the following question: does the difference in macrolide resistance rates arise from differences in regions or differences in

medical institutions? In Sapporo, both MS and MR *M. pneumoniae* were detected at medical institutions where *M. pneumoniae* was detected in more than 3 samples. In Asahikawa, there are 14 pediatric clinics and 5 outpatient pediatric clinics in hospitals. K Hospital and L Clinic, therefore, cover at least 10% of pediatric patients in Asahikawa. K Hospital, located in the western part of Asahikawa, is a secondary medical care center, and people living anywhere in the city visit this hospital. L Clinic is located in the eastern part of Asahikawa, and people living in the eastern part of the city visit this clinic. M Hospital in Kushiro, which participated in this study, is a secondary medical care center, and people living anywhere in the city visit this hospital. In Muroran, there are 2 pediatric clinics and 2 outpatient pediatric clinics in hospitals. N Clinic covers approximately 30% of pediatric patients living anywhere in Muroran (Table 1). Therefore, it is reasonable to assume that the bias toward MR (Kushiro) or MS (Muroran and Asahikawa) derives from regional differences, not from institutional differences.

MR *M. pneumoniae* was detected in 77.4% (48 of 62) of patients visiting hospitals and only in 9.1% (3 of 33) of patients visiting clinics; this pathogen was detected in 80.0% (20 of 25) of inpatients and only in 44.3% (31 of 70) of outpatients (Table 2). These differences can be at least partially explained by the fact that patients infected with MR *M. pneumoniae* have a fever of longer duration than do patients infected with MS *M. pneumoniae* (5.1 vs. 1.7 days, manuscript in preparation).

In agreement with our results (Table 3), it has been reported that pre-existing clones of MR *M. pneumoniae* can undergo selection and that the development of *de novo* macrolide resistance occurs during macrolide treatment (6,15). Even when the 25 patients who had received macrolides before collection of nasopharyngeal swab samples were excluded, statistical significance of regional differences was still observed: 0.0% (0 of 9) in Muroran, 5.6% (1 of 18) in Asahikawa, 38.5% (10 of 26) in Sapporo, and 100.0% (17 of 17) in Kushiro (Table 1), suggesting the importance of regional differences in the prevalence of MR *M. pneumoniae*. Unfortunately, because the number of patients infected with MR *M. pneumoniae* in Muroran and Asahikawa was too small and because clinics in Kushiro and hospitals in Muroran did not participate in this study, it was impossible to adjust the data for confounding factors by means of a statistical model. We therefore presented the crude prevalence rates for each category and for some combinations.

All MR *M. pneumoniae*-positive samples here have the A2063G mutation in the 23S rRNA gene. The origin of the A2063G mutation is another puzzle: does MR *M. pneumoniae* appear *de novo* or do a limited number of clones spread to various regions? The strains of MR *M. pneumoniae* isolated in Kushiro were found to have at least two origins, indicating that the spread of a single clone of MR *M. pneumoniae* could not account for the observed macrolide resistance (Table 5). Our study has several limitations. The number of patients was too small for full characterization of the prevalence of MR *M. pneumoniae*. In addition, the number of areas that participated in the study was limited. A surveillance program covering much broader areas is needed.

In conclusion, there are regional differences in the prevalence of MR *M. pneumoniae* infections in pediatric patients in Hokkaido. After exclusion of the patients who received macrolides before collection of nasopharyngeal swab samples, statistical significance of regional differences was still observed.

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Appendix The members of the Hokkaido Pediatric Respiratory Infection Study Group are as follows: Nobuhisa Ishiguro, Naoko Koseki, Miki Kaiho, Hideaki Kikuta, Takehiro Togashi, Keisuke Morita, Naoko Nagano, Masanori Nakanishi, Kyosuke Hazama, Toru Watanabe, Satoshi Sasaki, Tadashi Ariga, Akiko Okamura, Shigeru Yamazaki, Satoru Shida, Naofumi Kajii, Tetsuo Nagashima, Mikio Yoshioka, Yutaka Takahashi, Mutsuko Konno, Akihito Ishizaka, Takeyasu Takebayashi, Mutsuo Shibata, Hideto Furuyama, Hiroyuki Sawada, Yoshihiro Matsuzono, Mari Murashita, Tatsuru Yamanaka, Hiroyuki Naito, Yasushi Akutsu, Hayato Aoyagi, Katsuyuki Tobise, Chie Tobise, and Katsumi Azuma.

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